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Separation and quantitation of long-chain free fatty acids in human serum by high-performance liquid chromatography

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SUMMARY

A rapid, simple and highly sensitive reversed-phase high-performance liquid chromatographic method is described for the separation and quantitation of fatty acids in human serum using a very reactive fluorescent labeling reagent, 9-anthryldiazomethane. Quantitative esterification proceeds at room temperature without heat or catalysis. Baseline separation of nineteen select fatty acids from a standard mixture was achieved on two C_{18}-bonded silica columns connected in tandem using stepwise gradient elution of an acetonitrile–methanol–water mobile phase. The eluent was monitored by a fluorescence detector (maximum excitation wavelength, 365 nm; maximum emission wavelength, 412 nm). The procedure was applied to the analysis of both saturated and unsaturated long-chain free fatty acids (C_8 to C_{22}) extracted from human serum. Sera from fasting and non-fasting subjects were analyzed to show the applicability of this assay to biological samples. Detection limit and recovery of free fatty acids in serum were <10 pmol/μl and $>92\%$, respectively.

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INTRODUCTION

Most laboratories typically have relied upon gas chromatography for the separation and quantitation of fatty acids. Although the analysis of serum fatty acids by high-performance liquid chromatography (HPLC) has become increasingly popular, it is complicated by the lack of a strongly ultraviolet-absorbing chromophobe [1]. Consequently, over 47 different precolumn labeling agents have been developed to produce a suitable response for HPLC analysis of free fatty acids in biological samples. Despite such a large repertoire of derivatization agents, many existing methods suffer from excessively long run times [2] or require isolation of fatty acids with minicolumns [3]. A major advantage of the following method is that, unlike other derivatization protocols requiring heat and/or catalysis [4,5], it is useful for much more labile, easily oxidizable polyunsaturated fatty acids and their metabolites.

The purpose of this study was to develop a simple, precise and highly sensitive HPLC method to separate and quantitate long-chain fatty acids. The biological samples were extracted with chloroform–methanol and the complex mixture of C_8 to C_{22} fatty acids were esterified under neutral conditions, without heat or catalysis, using the fluorescent labeling agent 9-anthryldiazomethane (ADAM). The derivatized fatty acids were then fractionated on a reversed-phase C_{18}-bonded silica column by HPLC with a precisely programmed gradient of acetonitrile–methanol–water solvent system. Easily reproducible baseline separation of the nineteen select fatty acids was achieved. To demonstrate the applicability of this assay to biological samples, free fatty acid levels in sera from fasted and non-fasted human subjects were determined.

EXPERIMENTAL

Fatty acids used as standards were purchased from Sigma (St. Louis, MO, U.S.A.) and NuChek Prep (Elysian, MN, U.S.A.). ADAM was either obtained from Funakoshi (Tokyo, Japan) or synthesized in our laboratory. 9-Anthraldehyde (97%), potassium bromide (99%+, IR grade) and hydrazine hydrate (85%) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Nuclear 'SN' active carbon was obtained from Westvaco (Covington, VA, U.S.A.). [9,10-^{3}H(N)]Palmitic acid, [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid and [1-^{14}C]stearic acid were obtained from Amersham (Arlington Heights, IL, U.S.A.). All reagents and solvents were the highest grade quality commercially available.

Each fatty acid was dissolved in chloroform–methanol (2:1) to a concentration of 1 nmol per 10 μl and stored at -70°C in 16-ml Teflon-capped glass vials, flushed with nitrogen.

All melting point determinations of ADAM were obtained on a Fisher-Johns melting point apparatus (Springfield, NJ, U.S.A.) and are uncorrected. Infrared spectra were determined with KBr pellets using a Perkin Elmer Model

5999B spectrophotometer (Norwalk, CT, U.S.A.). Ultraviolet absorption spectra were obtained in toluene using a Gilford UV–VIS 2600 microprocessor-controlled system equipped with a thermal-programmed printer (Gilford, Oberlin, OH, U.S.A.) and a Hewlett-Packard 7225B graphics plotter (San Diego, CA, U.S.A.). Radioactivity measurements were performed on a Packard Tri-Carb 300C liquid scintillation system (Packard, Downers Grove, IL, U.S.A.) using automatic external standardization to correct for quenching.

Synthesis of ADAM was carried out as described by Nakaya et al. [6] using HgO and successfully crystallized in our laboratory by lyophilization. ADAM consists of brick-red crystals that can be stored, under dessication, at −70°C for more than six months if placed within a tightly sealed, small amber vial flushed with nitrogen. The identity of ADAM was confirmed by melting point (lit., 63–64°C) [6], ultraviolet spectrophotometry [7] and infrared spectrophotometry (KBr pellet: intense stretching vibration of the diazo N–N bond at 4831 cm^{-1}) [2].

HPLC apparatus

The chromatographic system (Perkin Elmer, Norwalk, CT, U.S.A.) consisted of a Series 3B HPLC digital solvent programmer and pumps, a 650-10LC variable-wavelength fluorescence spectrophotometer set at an excitation maximum of 365 nm (2 nm slit width) and an emission maximum of 412 nm (2 nm slit width) with a flow cell volume of 8 μl, a Xenon power supply (No. 150), a Sigma 14 chromatography data station and a WISP 710B automatic sample injector (Waters Assoc., Milford, MA, U.S.A.).

HPLC separation

HPLC separation was accomplished using two stainless-steel (15 cm×0.46 cm I.D. and 25 cm×0.46 cm I.D.) Zorbax octyldecylsilyl, 5 μm particle size, C_{18} reversed-phase analytical columns (DuPont, Wilmington, DE, U.S.A.) connected tandem. Column back-pressure varied from 10 to 14 MPa. A 5 cm×0.46 cm I.D. guard column was dry packed with Bondapak C_{18} Corasil, 37–50 μm pellicular column packing material (Waters Assoc.). Mobile phase solvents were: solvent A, acetonitrile; solvent B, methanol–water (1:1, v/v). The following gradient program was used: segment 1: isocratic at 90% A and 10% B for 20.0 min; segment 2: a linear gradient from initial conditions to 94% A and 6% B in 2.5 min; segment 3: isocratic at 94% A and 6% B for 12.5 min; segment 4: a linear gradient from preceding segment conditions to 100% A in 2.5 min; segment 5: isocratic at 100% A for 59.5 min. Columns were re-equilibrated to initial conditions (segment 1) for 15 min between each run. Flow-rate was 1.5 ml/min at ambient temperature (25°C). The solvent program was started simultaneously with the injection. Volume of sample injected was 50 μl. A silica precolumn was placed between the pump and the injector. Quanti-

tation of fatty acids were based on peak areas calculated electronically by the chromatography data station.

Decontamination procedures

Certain procedures were utilized to ensure a stable, reproducible and flat baseline. Nitrogen gas, used to dry down samples, was purified by connecting a 6.4-mm-diameter copper tubing from the tank and immersing a section of the tubing into a dry ice–acetone bath. Only acid-washed glassware and Teflon-capped vials were used; no soap, plastic pipette tips or rubber bulbs were utilized. Sample injection vials were thoroughly cleaned by the method of Spex industries [8]. Impurities in the HPLC-grade chloroform were eliminated by pretreatment with column chromatography [9].

Blood collection procedure

Healthy human volunteers in our laboratories (three males and two females, aged 22–45 years) provided both fasting and non-fasting serum samples. Subjects fasted for 12 h prior to the first blood collection and another sample was collected 3 h after eating. Non-heparinized blood samples were placed on ice, allowed to clot, centrifuged for 10 min at 1000 *g*, and the serum supernatant was frozen at $-70°C$ until assayed.

Free fatty acid extraction

Serum (75 μl) was pipetted into a 100 mm $\times$ 13 mm glass disposable culture tube and diluted with 400 μl of 0.5 *M* phosphate buffer (pH 6.4). An extraction solution (3.6 ml) of chloroform–methanol (2:1, v/v) and the internal standard solution (20 μl) of the heptadecanoic acid [2 nmol of $C_{17\ 0}$ per 20 μl of chloroform–methanol (2:1)] were added. $C_{17\ 0}$ was used as the internal standard because it is not present in human serum. The mixture was vortexed for 90 s and centrifuged for 2 min at 1000 *g*. Both the upper aqueous layer and the lipid interface layer were carefully suctioned off using an acid-washed glass pipette attached to a water aspirator. The tubes were recentrifuged for 3 min at 1000 *g*, and 2 ml of the lower organic layer were transferred to another 100 mm $\times$ 13 mm glass disposable culture test tube and evaporated to dryness under a stream of nitrogen gas in a 30°C water bath.

Derivatization procedure

ADAM stock solution (1 mg/ml) was prepared daily by dissolving the crystals in chloroform, followed by addition of methanol to reach the final chloroform–methanol concentration of 2:1 (v/v). ADAM crystals remain stable at room temperature during weighing but should be protected from light and returned to the freezer as soon as possible. The molar ratio of ADAM/free fatty acid was 41:1. The orange ADAM solution (200 μl) was added to the lipid residue in the test tube, mixed well, transferred to an autosampler injection

vial using a glass pipette and allowed to derivatize in the dark at room temperature for 8 h. The reaction of fatty acids with ADAM is over 90% complete within 3 h [10]. A 50-μl aliquot of this solution was injected directly into the liquid chromatograph without filtering the sample. Injections larger than 200 μl were dried down, redissolved in 10 μl of dimethylformamide and brought up to initial volume with chloroform–methanol (2:1, v/v).

RESULTS

A typical HPLC separation of a mixture containing both saturated and unsaturated derivatized fatty acids is shown in Fig. 1. Several characteristics of this reversed-phase separation should be noted. First, as the carbon number of fatty acids increases, the retention time also increases. Second, as the degree of unsaturation increases for a given carbon number, the retention time decreases. Third, weakly retained shorter-chain fatty acids require a higher water concentration (thus, less acetonitrile) in the mobile phase for fractionation. Retention times are shown in Table I.

The linearity of this analytical method was calculated. The correlation coef-

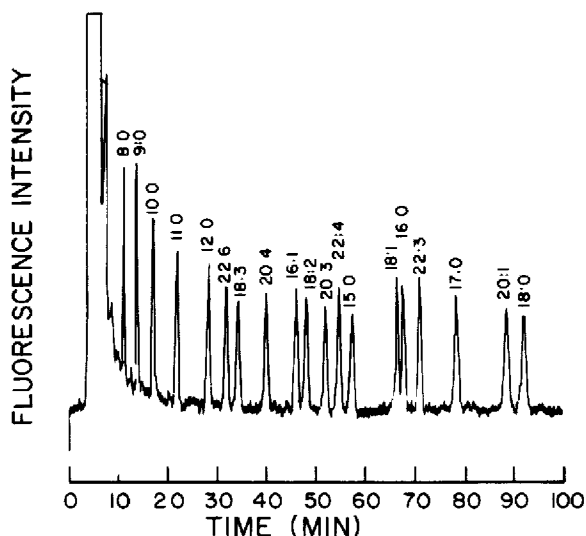

Fig. 1. HPLC separation of a select mixture of fatty acid ADAM derivatives containing 100 pmol of each fatty acid. Separation was performed on two octyldecylsilyl columns in tandem with a stepwise gradient of solvent A (acetonitrile) and solvent B (methanol–water, 1:1, v/v) at ambient temperature and a flow-rate of 1.5 ml/min. The following linear gradient was used: Segment 1: 90% A and 10% B for 20 min; segment 2: initial conditions changed to 94% A and 6% B over 2.5 min; segment 3: 94% A and 6% B for 12.5 min; segment 4: preceding conditions changed to 100% A over 2.5 min; segment 5: 100% A for 59.5 min. Fluorescence detector: excitation at 365 nm; emission at 412 nm. Injection volume 50 μl.

TABLE I

RETENTION TIMES OF ADAM ESTERS OF VARIOUS FATTY ACIDS USING REVERSED-PHASE HPLC ($n = 15$)

Fatty acid	Abbreviation	Retention time (min)
Octanoic acid	$C_{8\ 0}$	11 47
Nonanoic acid	$C_{9\ 0}$	14 19
Decanoic acid	$C_{10\ 0}$	17.84
Undecanoic acid	$C_{11\ 0}$	22.80
Dodecanoic acid	$C_{12\ 0}$	29 60
cis-4,7,10,13,16,19-Docosahexaenoic acid	$C_{22\ 6}$	33.42
cis-9,12,15-Octadecatrienoic acid	$C_{18\ 3}$	35.48
cis-5,8,11,14-Eicosatetraenoic acid	$C_{20\ 4}$	39.91
cis-9-Hexadecenoic acid	$C_{16\ 1}$	45 98
cis-9,12-Octadecadienoic acid	$C_{18\ 2}$	48.03
cis-11,14,17-Eicosatrienoic acid	$C_{20\ 3}$	51.01
cis-7,10,13,16-Docosatetraenoic acid	$C_{22\ 4}$	52.77
Pentadecanoic acid	$C_{15\ 0}$	55.03
cis-9-Octadecenoic acid	$C_{18\ 1}$	62.46
Hexadecanoic acid	$C_{16\ 0}$	64.23
cis-13,16,19-Docosatrienoic acid	$C_{22\ 3}$	67.10
Heptadecanoic acid	$C_{17\ 0}$	75.80
cis-11-Eicasenoic acid	$C_{20\ 1}$	86.19
Octadecanoic acid	$C_{18\ 0}$	90.54

ficient ranged from 0.996 to 0.998 between 20 and 2000 pmol for all fatty acids. These results indicate that this derivatization procedure is suitable for quantitative purposes. The practical lower limit of detection in serum was < 10 pmol/μl.

For the recovery studies, a standard mixture of fatty acids (2 nmol of each fatty acid) was derivatized and compared to the peak areas obtained with 2 nmol of each fatty acid were added to 75 μl of serum and subsequently extracted. Recovery of each free fatty acid was $> 92\%$ with and without serum. In addition, efficiency of solvent extraction was also determined by comparing the recovery of $[^3H]C_{16\ 0}$, $[^3H]C_{20\ 4}$ and $[^{14}C]C_{18\ 0}$ with and without serum. Recovery of these radioactive fatty acids was $> 96\%$ with and without serum.

The precision of the method also was determined for each fatty acid when 50–2000 pmol were injected. The coefficients of variation (C.V.) ranged from 6.3 to 8.9% (intra-assay variability) and from 8.9 to 12.7% (inter-assay variability). For ≤ 20 pmol injected, the C.V. rose to $> 20\%$.

A representative chromatogram of serum free fatty acids obtained from a normal fasted human subject is shown in Fig. 2. Similar chromatograms were also obtained from fed human subjects. Positive peak identification for each free fatty acid in serum was achieved by comparing the retention time with that of the corresponding standard fatty acid derivative. All standards were

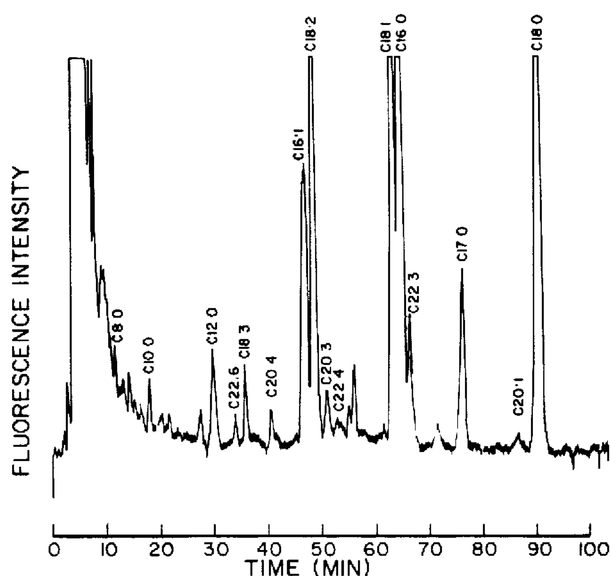

Fig. 2. HPLC separation of free fatty acids found in 75 μl normal fasted human serum. The separation was performed under the chromatographic conditions described in Fig. 1.

TABLE II

AVERAGE SERUM CONCENTRATIONS OF FREE FATTY ACIDS IN BOTH FASTED AND FED HUMAN SUBJECTS

Free fatty acid[a]	Fed subjects		Fasted subjects	
	Concentration (mean $\pm$ S.D., $n=5$) (nmol/ml)	Percentage of total	Concentration (mean $\pm$ S.D., $n=5$) (nmol/ml)	Percentage of total
$C_{8\,0}$	0.1 $\pm$ 0.1	(0.1)	0.3 $\pm$ 0.2	(0.1)
$C_{10\,0}$	1.2 $\pm$ 0.6	(0.7)	1.2 $\pm$ 0.09	(0 4)
$C_{12\,0}$[b]	3.2 $\pm$ 1.5	(1.8)	5.8 $\pm$ 0.6	(1 9)
$C_{22\,6}$	0 9 $\pm$ 0.3	(0.5)	2.3 $\pm$ 1.2	(0.8)
$C_{18\,3}$	2 3 $\pm$ 1.0	(1.3)	3.9 $\pm$ 1.1	(1.3)
$C_{20\,4}$[c]	1.7 $\pm$ 0.4	(1.0)	4.7 $\pm$ 1.4	(1 6)
$C_{16\,1}$[c]	11.8 $\pm$ 4.2	(6.7)	18 5 $\pm$ 1 9	(6 2)
$C_{18\,2}$	30.7 $\pm$ 6.8	(17.6)	52.1 $\pm$ 15.5	(17.4)
$C_{20\,3}$[c]	0.9 $\pm$ 0.6	(0.5)	2.4 $\pm$ 0.8	(0.8)
$C_{22\,4}$[b]	0.3 $\pm$ 0.1	(0.2)	1.0 $\pm$ 0.5	(0.3)
$C_{18\,1}$	59.2 $\pm$ 16.2	(33.9)	93 7 $\pm$ 38.9	(31 3)
$C_{16\,0}$[b]	43.5 $\pm$ 9 1	(24.9)	80.7 $\pm$ 26.4	(27.0)
$C_{22\,3}$	0.04 $\pm$ 0.04	(0.02)	0.1 $\pm$ 0.08	(0.03)
$C_{20\,1}$	0.7 $\pm$ 0.08	(0 4)	0.9 $\pm$ 0.6	(0.3)
$C_{18\,0}$[b]	18.1 $\pm$ 3.3	(10.4)	31.5 $\pm$ 7.8	(10.5)
Total	174.9 $\pm$ 38.8	(100.0)	299.0 $\pm$ 90.2	(100 0)

[a]Fatty acids are expressed as the number of carbon atoms: number of double bonds.
[b,c]The level of significance between the two groups was calculated using the two-tailed Student's t-test ([b]$p<0.05$; [c]$p<0.02$).

chromatographed individually and in various mixtures. In addition, the proposed method provides a well resolved separation of $C_{20\ 4}$, $C_{22\ 3}$, $C_{22\ 4}$ and $C_{22\ 6}$ from the other free fatty acids in serum.

As shown in Table II, the concentrations of some free fatty acids were significantly increased in fasted serum compared to non-fasted serum. Especially in the fasted state, highly unsaturated free fatty acids, such as $C_{22\ 6}$, $C_{20\ 4}$, $C_{20\ 3}$, $C_{22\ 4}$ and $C_{22\ 3}$, increased from 2.5-fold to 3.3-fold compared to the fed state. Under conditions of fasting, several of these polyunsaturated fatty acids, which are derived from the hydrolysis of membrane phospholipids and triglycerides, serve as critical precursors to numerous eicosanoid metabolites.

DISCUSSION

Current interest in the metabolism of fatty acids necessitates the development of a highly sensitive method for their determination in biological samples. However, the analysis of fatty acids is hampered by the lack of a strongly ultraviolet-absorbing or fluorescent chromophobe. Thus, the majority of carboxylic acid-containing compounds of biochemical interest are typically derivatized with ultraviolet-absorbing or fluorescent compounds. One example of an increasingly common reagent is ADAM, which already has been applied to the determination of prostaglandins [11], biotin [12], 5,8,11,14,17-eicosapentaenoic acid [13], bile acids [10], dicarboxylic acids such as oxalic acid [14], short- and medium-chain carboxylic acids [15,16] and amino acids [17]. As evidenced by these findings, the reactivity of ADAM does not seem to be comprised by its aryl nature, and steric hindrance does not seem to play a role in the derivatization reaction.

The present study offers considerable improvements over existing analyses for many reasons. First, the marked and specific reactivity of ADAM is ideal for derivatizing carboxylic acids, such as polyunsaturated fatty acids which are labile to excessive heating, acidic or basic conditions. Second, ADAM yields a fluorescent product that possesses a much higher degree of sensitivity, selectivity and specificity than an ultraviolet-absorbing product. Third, unlike some carboxylic labeling reagents, such as 4-bromethyl-7-acetoxycoumarin [18], ADAM does not require post-column alkaline hydrolysis to yield fluorescent derivatives. Fourth, almost any aprotic solvent may be used in the derivatization procedure. Fifth, it is unnecessary to remove excess derivatizing agent from the reaction mixture because any excess reagent is eluted within the first 10 min of the HPLC run.

Another major advantage of this proposed HPLC method compared to other methods is the relative ease that optimum separation is achieved for a greater number of fatty acids than previously reported [15]. Due to the high hydrophobicity of ADAM ester derivatives, best resolution was accomplished using two C_{18} octyldecylsilyl columns. It was also observed that separation of fatty

acids can be quite sensitive to the type of solvent system utilized. For example, it was extremely difficult to resolve $C_{16:0}$ and $C_{18:1}$ using a methanol–water system [10]. A large number of solvent combinations were tried in both isocratic and gradient modes in order to separate these coeluting peaks. Also various phosphate and silver salts, changing the pH and temperature conditions were tried. It was finally found that using an acetonitrile–methanol–water mobile phase provided the best resolution, highest efficiency and timely separation of fatty acids. Incorporation of acetonitrile definitely facilitated the separation of both unsaturated and saturated fatty acids. According to Glajch and Kirkland [19], in order to optimize mobile phase selectivity, the addition of a proton donor solvent (such as acetonitrile) to a proton acceptor solvent (such as methanol) leads to improved resolution primarily due to hydrogen bonding and dipole–dipole interactions. Moreover, Nimura and Kinoshita [15] have demonstrated that fluorescence intensity of esters is higher using an acetonitrile–water system than a methanol–water system.

Applicability of this method to the analysis of long-chain free fatty acids in a biological system is shown in this communication. Both the absolute as well as the relative free fatty acid levels (as a percentage of the total concentration) agrees well with other published papers [10, 20–22]. The results of our study clearly show that the total free fatty acid content in fasted human serum is about 1.7-fold higher than in fed human serum. This could mean that fasting causes catabolism of biological membranes.

Both the ease of preparation of ADAM and the excellent chromatographic properties of the resultant derivatives in reversed-phase HPLC provide a reliable, non-destructive and highly sensitive means to detect small amounts of compounds in biological samples.

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